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THE LIPID COMPOSITION OF PLASMA MEMBRANE AND MITOCHONDRIAL FRACTIONS FROM EPIDIDYMAL ADIPOCYTES OF COLD-ACCLIMATED RATS

GISELE CHERQUI, MONIQUE CADOT, CLAUDE SENAULT and RENE PORTET

*Laboratoire d'Adaptation Energétique à l'Environnement *, Ecole Pratique des Hautes Etudes, Collège de France, 11, Place M. Berthelot, 75231 Paris Cedex 05 (France)*

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Summary

1. The effects of cold acclimation (5°C) on the lipid composition of plasma membrane and mitochondrial fractions from epididymal adipocytes of rats were studied.

2. The adipocyte plasma membrane fraction of the cold-acclimated rats had lower lipid, phospholipid and cholesterol to protein weight ratios, a lower cholesterol to sphingomyelin molar ratio, and a higher linoleic acid content in the phospholipids than controls.

3. The mitochondrial fraction of the cold-acclimated rat adipocyte had lower ratios of cholesterol to protein (weight), to phospholipid and to cardiolipin (molar), and less sphingomyelin content than did controls.

4. These data, discussed in terms of alterations in physical and biochemical properties, indicate cold-induced changes at the membrane level in rat epididymal adipocytes.

Introduction

Rats exposed to low temperature (5°C) for periods of more than 3—4 weeks adapt to living in the cold and maintaining a high metabolic rate [1]. The main change in the cold-acclimated rat is the replacement of shivering thermogenesis by non-shivering thermogenesis [2], which occurs primarily in brown adipose tissue and muscle. Although white adipose tissue is not directly involved in

* Equipe de Recherches associée au CNRS No. 070413.

non-shivering thermogenesis, cold-acclimated epididymal adipose tissue has been shown to undergo morphological, constitutional and metabolic modifications. These changes include decreases in adipocyte cell size, and increases in cell number [3,4], in protein content [5,6] and in DNA and RNA contents [7], decreases in lipid content [3,8], increases in metabolic activity [9,10] and, especially, in the lipolytic response to norepinephrine (3,4,5) which is accounted for by increased norepinephrine-stimulated adenylate cyclase activity [11,12].

The present study was designed to investigate the effects of 4 weeks' cold-exposure on the lipid composition of the rat adipocyte plasma membrane and mitochondrial fractions.

Materials and Methods

Animals and diets

All the experiments were performed on 7-week-old male Long Evans rats acclimated to constant temperatures of either 28°C for 3 weeks (control group) or 5°C for 4 weeks (cold-acclimated group). Under this schedule the mean body weights in the two groups were approximately the same at the time of the experiments (280—300 g). The animals were maintained on a daily 12 h dark-light cycle and fed a standard laboratory diet and water ad libitum until killing. The fatty acid composition of the diet was analyzed periodically to ensure that it remained constant throughout the experiment.

Fractionation procedures

Isolated fat cells. The two halves of the epididymal fat pads taken from 9—12 decapited rats were pooled and isolated fat cells were prepared according to the procedure of Rodbell [13]. There were minor modifications: the absence of glucose; lowering of the collagenase concentration (5 mg/g adipose tissue); and decrease in the dissociation period (45 min).

Preparation of adipocyte plasma membranes. Free adipocytes were disrupted by the method of Avruch and Wallach [14]. The membranes were prepared according to the procedure of Laudat et al. [15] by isopycnic centrifugation on a linear sucrose density gradient (27.6—54.1% w/w) without ATP. This resulted in the separation of three bands at relative densities of 1.13—1.14 (plasma membrane fraction No. 1), 1.16—1.18 (mitochondrial fraction) and 1.25 (nuclear fraction).

Preparation of mitochondria. The mitochondrial fraction from the linear sucrose density gradient used for the plasma membrane preparation was removed and diluted with 8 vols. of 5 mM Tris-HCL, pH 7.4, and centrifuged at 35 000 $\times g$ for 15 min. The resulting pellet was resuspended in 12 ml of medium I (10 mM Tris-HCl/1 mM EDTA/0.25 M sucrose, pH 7.4). 6-ml portions were layered carefully on two 24-ml linear sucrose gradients and centrifuged as described by Laudat et al. [15]. Two particulate bands, at relative densities of 1.13—1.14 and 1.16—1.18, were separated. Each band was removed and the 1.13—1.14 band was diluted 8 : 1 (v/v) with 5 mM Tris-HCl, pH 7.4 and centrifuged. This was the plasma membrane fraction No. 2, which was then mixed with the plasma membrane fraction No. 1 previously prepared. The

1.16—1.18 band was treated similarly and the pellet was resuspended in 1.5 ml of 5 mM Tris-HCl, pH 7.4; it constituted the 'purified' mitochondrial fraction.

Characterization of subcellular fractions

Electron microscopy. Aliquots of plasma membrane and mitochondrial fractions were pelleted and immediately fixed by 2% osmic acid in phosphate buffer (pH 7.3). Electron microscopic examinations were kindly performed by Dr. Vodovar (INRA, Jouy en Josas, France).

5'-Nucleotidase assay (EC 3.1.3.5). The assay system in a final volume of 1.25 ml contained 1.13 ml 5 mM AMP in 20 mM barbital buffer (pH 7.5), 10—15 μg of protein fraction, and glass-distilled water. Incubations were carried out in duplicate for 30 min at 37°C in a water bath. Zero time controls had the fraction aliquots added to the complete reaction mixture maintained at 4°C and containing 0.25 ml of 30% trichloroacetic acid. The reaction was stopped by adding 0.25 ml of cold 30% trichloroacetic acid, and the mixture was allowed to stand at 4°C for 5 min and then centrifuged. The precipitate was sedimented and a 1-ml aliquot of the supernatant was assayed for inorganic phosphate according to the method of Fiske and Subbarow [16].

Proteins

Proteins were determined by the method of Lowry et al. [17]. To avoid interference by sucrose, proteins were precipitated by the addition of 200 μl of 100% perchloric acid. The samples were centrifuged and proteins were determined after solubilization with 0.1 M NaOH in comparison to a serum albumin standard prepared in a similar manner.

Lipid analysis

The total lipids were extracted according to the procedure of Folch et al. [18] as modified by Comte et al. [19].

Aliquots of extract were analyzed for phospholipid phosphorus using the Bartlett method [20].

After hydrolysis and selective precipitation by digitonin as described by Sperry and Webb [21], additional aliquots were analyzed for cholesterol content by the technique of Zak [22].

Two-dimensional chromatography on 20 × 10 cm plates coated with silica gel H following the procedure of Zwingelstein et al. [23] was used to separate the different classes of phospholipid from chloroform/methanol extracts. Individual phospholipids were identified with A grade reference standards (Koch-Light).

Phospholipids were separated from neutral lipids by one-dimensional thin-layer chromatography (20 × 10 cm plates coated with silica gel G) using ethyl ether as eluant. The phospholipid and neutral lipid zones were scraped and recovered from the silica gel by extraction with chloroform/methanol, 1 : 1 (v/v). The methyl esters were prepared by transesterification by heating 0.5—1 mg of lipid with 1 ml of sulfuric methanol (one drop H_2SO_4 per 1 ml methanol) in a sealed glass tube for 3 h at 100°C. After neutralization by 1 ml 5% potassium carbonate, the methyl esters were extracted into pentane. The fatty acid composition was determined using a Hewlett Packard gas chromatograph with

a 6 ft. × 0.12 inch metallic column filled with chromosorb W coated with 15% diethyleneglycol succinate. Fatty acids were identified by using standards obtained from Applied Science Laboratories.

Reagents

Crude collagenase (200 units/mg) from *clostridium histolyticum* was obtained from Worthington; bovine serum albumin fraction V poor in fatty acids from Sigma; Silica Gel G (Kieselgel 60 PF 254) from Merck; and Silica Gel H (Kieselgel N-HR) from Macherey Nagel.

Results

Characterization of the subcellular fractions

Electron microscopy. The plasma membrane fraction consisted primarily of membrane vesicles of different sizes. Flask-shaped invaginations which have been shown to be characteristic of plasma membranes of fat cell [24] were observed in some portions of the larger vesicles. In some fields, occasional ribosomes attached to membranes were noted. No components originating from mitochondria were seen.

Ultrastructural appearance of mitochondrial fraction from the cold-acclimated group (Fig. 1B) is extensively modified by comparison to control group (Fig. 1A).

Purity of the fractions. The purity of the membrane fraction was assessed by comparing the 5′-nucleotidase specific activity in the plasma membrane with that of the homogenate (25 000 × *g* pellet suspended in medium I as described by Laudat et al. [15]). This activity was enriched 3- to 4-fold in the isolated plasma membranes of both groups (Table I). Approximately 50% of the total enzymatic activity recovered from the three fractions separated on sucrose gradient (about 112% of the homogenate activity) was found in the plasma membrane fraction.

The contamination of the plasma membrane fraction by phosphorus originating from nucleic acids was evaluated by the ratio, lipid phosphorus/total phosphorus. 93% of the phosphorus content of the plasma membranes was accounted for by phospholipids.

The absence of contamination of the plasma membrane fraction by the mitochondria, revealed by electron microscopy, was also confirmed by the absence of cardiolipin.

The purity of the mitochondrial fraction was indicated by the loss of 5′-nucleotidase activity. Upon rehomogenizing the mitochondrial zone isolated on the first density gradient and recycling it through an identical sucrose gradient, we observed a 43% decrease in 5′-nucleotidase activity, which was released into a density region corresponding to that of plasma membranes (1.13—1.14). As this latter fraction showed a 5′-nucleotidase specific activity equivalent to that of the plasma membrane fraction isolated on the first sucrose density gradient, it appeared that the decrease in specific activity did not result from the inactivation of the 5′-nucleotidase but from an actual loss of the enzyme.

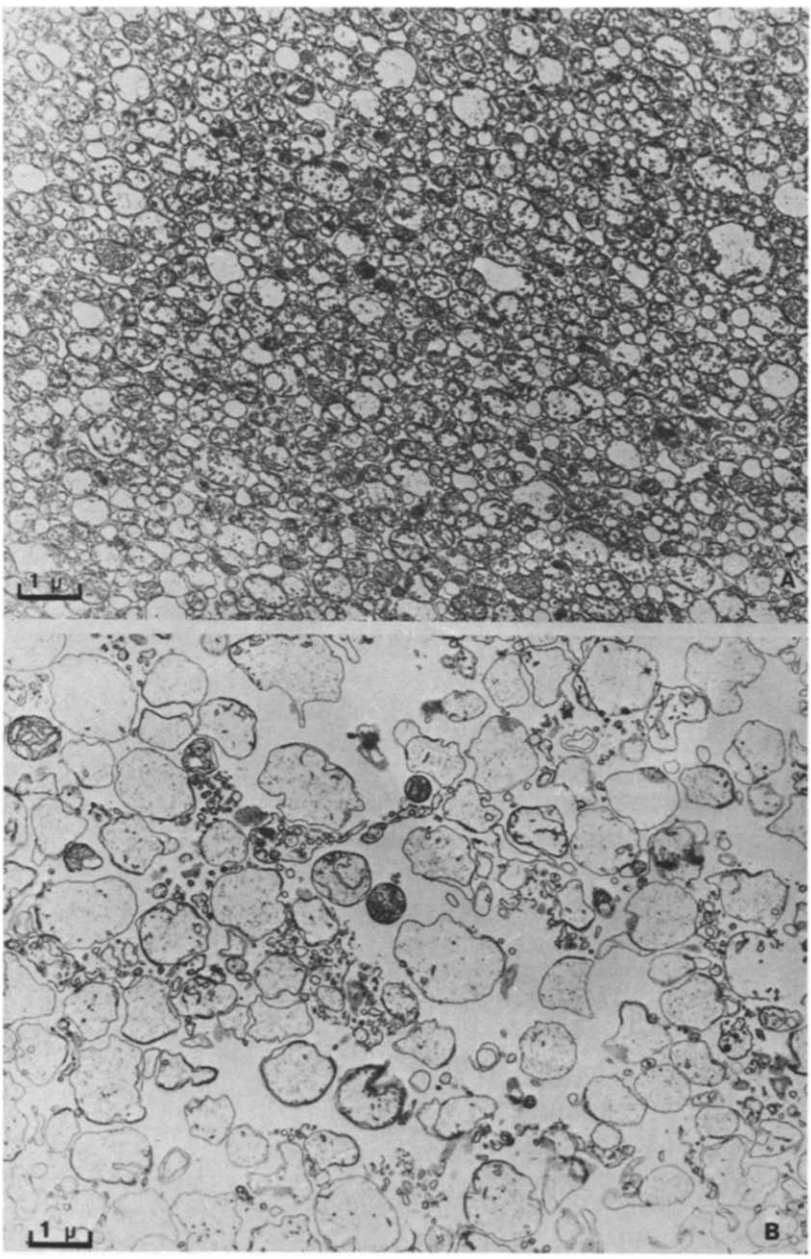

Fig. 1. Ultrastructural appearance of mitochondrial fractions from epididymal adipocytes of control (28°C) and cold-acclimated rats (5°C). The mitochondrial fraction from control rats (A) consisted of whole mitochondria showing double membranes and the typical cristae. The mitochondrial fraction from cold-acclimated rats (B) consisted primarily of extensively swollen organelles, showing a clear and granular matrix; most of the cristae had disappeared. Loss of the outer membrane was noted in some mitochondria.

TABLE I

5′-NUCLEOTIDASE SPECIFIC ACTIVITY IN ADIPOCYTE PLASMA MEMBRANE FRACTIONS PREPARED BY SUCROSE DENSITY GRADIENT

Enzyme assay was done at 37°C. Values are the mean ± S.E. Numbers in parentheses represent the number of preparations analyzed.

5′-Nucleotidase	28°C (6)			5°C (4)		
	Spec. act. (nmol · P_i/mg protein per min)	Recovery * (%)	Purification (RSA) **	Spec. act. (nmol · P_i/mg protein per min)	Recovery * (%)	Purification (RSA) **
Homogenate	16.5 ± 3.4			16.2 ± 2.2		
Plasma membrane fraction	67.6 ± 7.5	46.3	4.3	50.8 ± 12.2	49.0	3.1

* In percent of the total activity recovered in the three fractions separated on sucrose density gradients.
** RSA, relative specific activity, is the ratio of specific activity of enzyme in the plasma membrane fraction to the specific activity in the homogenate.

Lipid composition

Plasma membranes. The chemical composition of adipocyte plasma membranes from control and cold-acclimated rats is shown in Table II. The protein content of cold-acclimated adipocyte plasma membranes was increased by 12%, and the phospholipid and cholesterol contents were decreased, respectively, by 11% and 22%. Adipocyte plasma membranes from the cold-acclimated rats contained lower ratios of lipid, phospholipid and cholesterol to protein (weight) than did controls. No difference was noted in the cholesterol to phospholipid molar ratio.

Analysis of the phospholipid composition of the adipocyte plasma mem-

TABLE II

CHEMICAL COMPOSITION OF ADIPOCYTE PLASMA MEMBRANE AND MITOCHONDRIAL FRACTIONS FROM CONTROL (28°C) AND COLD-ACCLIMATED RATS (5°C)

The plasma membrane and mitochondrial fractions were prepared and the lipids analyzed as described in Materials and Methods. Values are the mean ± S.E. The total weight (wt.) was calculated from the sum of protein and lipid contents. Lipid content is the sum of phospholipid and cholesterol contents. Numbers in parentheses are the number of preparations analyzed.

	Plasma membrane fraction		Mitochondrial fraction	
	28°C (4)	5°C (7)	28°C (4)	5°C (6)
Protein content (% by wt.)	51.0 ± 0.3	57.0 ± 0.3 ***	70.4 ± 2.9	69.0 ± 1.7
Lipid content (% by wt.)	48.9 ± 0.5	42.9 ± 0.6 ***	29.6 ± 2.4	30.9 ± 0.4
Phospholipid content (% by wt.)	41.2 ± 0.5	36.8 ± 0.7 **	26.9 ± 2.2	29.2 ± 0.6
Cholesterol content (% by wt.)	7.7 ± 0.2	6.0 ± 0.3 **	2.6 ± 0.3	1.7 ± 0.1 *
Lipid/protein (w/w)	0.96 ± 0.02	0.75 ± 0.02 ***	0.42 ± 0.05	0.45 ± 0.01
Phospholipid/protein (w/w)	0.81 ± 0.01	0.65 ± 0.01 ***	0.39 ± 0.04	0.42 ± 0.01
Cholesterol/protein (w/w)	0.15 ± 0.02	0.11 ± 0.01 **	0.038 ± 0.004	0.025 ± 0.001 **
Cholesterol/phospholipid (molar ratio)	0.38 ± 0.01	0.33 ± 0.02	0.20 ± 0.02	0.13 ± 0.01 **

* Statistically different from the control group; $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

branes from control and cold-acclimated rats (Table III) indicated similar phospholipid profiles. However, a decrease of 33% and 29% was observed in the cholesterol to sphingomyelin and in the cholesterol to phosphatidylcholine molar ratios respectively, in the membranes of cold-acclimated rats.

The fatty acid composition of the phospholipids is shown in Table IV. Similar amounts of saturated and monoenoic acids were present in plasma membrane preparations of both groups. The only difference was in the linoleic acid (C-18 : 2) content which was increased by 29% in the adipocyte plasma membranes of cold-acclimated rats. No difference was noted in the ratio, number of double bonds/phospholipid (molar), which served as an index of unsaturation.

Mitochondrial fraction. As shown in Table II, in both experimental groups, mitochondria had higher protein content and lower lipids content than plasma membranes. Cold acclimation did not change the amounts of protein and phospholipid in adipocyte mitochondria but decreased the cholesterol content by 35%. The ratios of cholesterol to protein (weight) and cholesterol to phospholipid (molar) were respectively decreased by 34% and 35% in cold-acclimated adipocyte mitochondria.

The phospholipid composition of the mitochondria is shown in Table III. Mitochondria differed from plasma membranes by the presence of cardiolipin and their lower sphingomyelin content. The sphingomyelin content of the cold-acclimated adipocyte mitochondrial fraction was 33% lower than controls. Since cholesterol is primarily associated with the outer membrane [25] and cardiolipin exclusively associated with the inner membrane [26] the cholesterol/cardiolipin molar ratio was calculated. An important decrease in this ratio (44%) was observed in the cold-acclimated adipocyte mitochondrial fraction.

TABLE III

PHOSPHOLIPID COMPOSITION OF ADIPOCYTE PLASMA MEMBRANE AND MITOCHONDRIAL FRACTIONS FROM CONTROL (28°C) AND COLD-ACCLIMATED RATS (5°C)

The phospholipids were separated as described in Materials and Methods. Values (percentages by weight) are the mean ± S.E. Numbers in parentheses are the number of preparations analyzed.

	Plasma membrane fraction		Mitochondrial fraction	
	28°C (5)	5°C (4)	28°C (5)	5°C (4)
Phosphatidylethanolamine	28.1 ± 1.7	24.6 ± 1.8	26.7 ± 0.8	28.4 ± 0.8
Phosphatidylcholine	39.5 ± 2.0	43.0 ± 1.5	44.3 ± 1.4	47.3 ± 1.9
Sphingomyelin	17.6 ± 1.8	18.7 ± 0.3	11.0 ± 0.7	7.4 ± 0.3 **
Phosphatidylinositol	7.0 ± 1.0	6.8 ± 1.1	5.6 ± 0.6	5.1 ± 1.0
Phosphatidylserine	7.7 ± 0.9	6.6 ± 0.5	4.9 ± 1.5	2.5 ± 0.5
Cardiolipin			7.5 ± 0.6	9.3 ± 1.1
Cholesterol/phosphatidylcholine (molar ratio)	1.0 ± 0.1	0.7 ± 0.1 (3) *		
Cholesterol/sphingomyelin (molar ratio)	2.4 ± 0.2 (3)	1.6 ± 0.1 (3) *		
Cholesterol/cardiolipin (molar ratio)			5.0 ± 0.4 (2)	2.8 ± 0.4 *

* Statistically different from the control group; $P < 0.05$.
** $P < 0.001$.

TABLE IV

FATTY ACID COMPOSITION OF ADIPOCYTE PLASMA MEMBRANE AND MITOCHONDRIAL PHOSPHOLIPIDS FROM CONTROL (28°C) AND COLD-ACCLIMATED (5°C) RATS

Values are the mean ± S.E. Numbers in parentheses indicate the number of determinations.

Fraction	Temperature (°C)	Fatty acid (mol %)							Fatty acid double bonds/ phospholipid (molar ratio)
		C-14 : 0	C-16 : 0	C-16 : 1	C-18 : 0	C-18 : 1	C-18 : 2	C-20 : 4	
Plasma membrane	28 (3)	1.9 ± 0.8	22.9 ± 3.8	2.8 ± 0.3	25.3 ± 1.4	15.3 ± 1.4	14.6 ± 1.4	17.0 ± 2.6	2.3 ± 0.2
	5 (4)	2.0 ± 0.5	19.1 ± 2.2	2.4 ± 0.1	25.2 ± 0.4	14.7 ± 0.3	18.9 ± 0.6 *	17.3 ± 1.9	2.5 ± 0.1
Mitochondrial	28 (3)	1.8 ± 0.1	22.3 ± 1.3	2.7 ± 0.2	23.9 ± 1.5	14.3 ± 0.8	18.1 ± 1.0	16.7 ± 0.7	2.4 ± 0.1
	5 (3)	1.5 ± 0.1	18.0 ± 2.4	2.9 ± 0.2	21.6 ± 1.3	14.6 ± 0.1	24.5 ± 2.6	16.9 ± 0.9	2.7 ± 0.1

* Statistically different from the control group; $P < 0.05$.

The fatty acid composition of phospholipids from the control and cold-acclimated adipocyte mitochondrial fractions is shown in Table IV. No difference was noted between the two groups; the C-18 : 2 increase (35%) observed for the cold-acclimated group was not statistically significant.

Discussion

The validity of using 5′-nucleotidase as a marker for adipocyte plasma membranes has been discussed by Avruch and Wallach [14], who concluded that it was a better marker than the ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). The ratios of the specific activity of this enzyme in the membrane preparation to that in the homogenate used to calculate membrane purification, showed comparable purification for both experimental groups (relative specific activity = 3—4). These values agree with the results of Avruch and Wallach [14] and Laudat et al. [15]. The recovery of 5′-nucleotidase in the plasma membrane fraction (Table I), which was similar in both groups, was lower than that reported by Avruch and Wallach (76%) [14]. This difference may be accounted for by the fact that these authors recovered only 85% of the total homogenate activity while we recovered more than 100% (approx. 112%), as observed by Kawai and Spiro (120%) [27]. The excess might be attributed either to experimental procedures or to inhibition of the 5′-nucleotidase activity by the EDTA present in the homogenate [28].

Small amounts of non-lipid phosphorus (7%) in the plasma membrane fractions could be accounted for by the presence of occasional ribosomes seen in electron microscope fields. However, such contamination may be overestimated since the presence of RNA in rat and in rabbit adipocyte plasma membranes is questionable [14,27,29].

The level of contamination of the plasma membranes by endoplasmic reticulum membranes has not been evaluated by enzymatic markers. Glucose-6-phosphatase (EC 3.1.3.9), a typical endoplasmic marker in liver, could not be detected in white adipose tissue [30]: moreover, the use of NADH oxidase (EC 1.2.4.6) may not be appropriate, since it has been suggested that there might be a NADH oxidase activity intrinsic to the plasma membrane [14,29, 30].

The lipid/protein, phospholipid/protein and cholesterol/protein weight ratios and the cholesterol/phospholipid molar ratio determined for the control group are in agreement with the values observed by other investigators for adipocyte plasma membranes [27] and liver plasma membranes [31—33].

Asworth and Green [34] have indicated that an increased membrane activity is associated with a decrease in the ratio of phospholipid/protein, parallel to an increased protein content involved in enzymatic and transducer functions. The decrease of this ratio observed in cold-acclimated adipocyte plasma membrane might also reflect an increased membrane activity.

The decrease in cholesterol content might be associated with an increased permeability of the cold-acclimated adipocyte plasma membrane. Bruckdorfer et al. [35] have observed that partial removal of cholesterol from erythrocyte ghosts increases their permeability to glycerol. Similar evidence is provided by

studies of liposomes in which a higher content of cholesterol is associated with decreased permeability [36].

The phospholipid composition of the control and cold-acclimated adipocyte plasma membranes in our experiments fell within the range of values reported by Maude et al. [37]. The decrease of the cholesterol/sphingomyelin molar ratio might reflect a poorer structural organization of the cold-acclimated adipocyte plasma membranes parallel to a decreased microviscosity [38]. Studies of cholesterol-sphingomyelin liposomes suggest that cholesterol forms specific complexes with sphingomyelin [38] and that these complexes form important membrane unit structures [39].

The fatty acid composition of the phospholipids in the control adipocyte plasma membrane fell within the range of values reported by R. Counis (personal communication). There was an increased linoleic acid content in the cold-acclimated adipocyte plasma membranes which may reflect an increased fluidity allowing for greater metabolic activity.

The purification of mitochondria was monitored by the loss of 5'-nucleotidase activity. The decrease in this activity following the purification (described in Materials and Methods) is in agreement with the data of Avruch and Wallach [14]. The swelling properties of cold-acclimated adipocyte mitochondria, as indicated by electron micrographs, may be related to an increased permeability which is accounted for by the chemical changes already presented. The major differences between the adipocyte mitochondrial fractions from the two groups were the lower sphingomyelin and cholesterol contents of the cold-acclimated group. The decreased cholesterol content which led to lower ratios of cholesterol to protein, to phospholipid and to cardiolipin could be related to an increased mitochondrial permeability [36].

The phospholipid composition of the control adipocyte mitochondrial fraction fell within the range observed by Gerson [40] in outer mitochondrial membranes from rat liver. Phospholipid composition obtained by Dod and Gray is different [41]. The decreased sphingomyelin content of cold-acclimated adipocyte mitochondria in addition to the decreased cholesterol/phospholipid molar ratio, suggests a lower microviscosity of the mitochondrial membranes [38].

The micrographs showed that some cold-acclimated adipocyte mitochondria had lost their outer membrane. This suggests a greater fragility of these organelles, a possibility supported by previous works on cold-acclimated rat liver mitochondria [42,43]. Another analogy with liver mitochondria of cold-acclimated rats can be found since we have observed a somewhat greater proportion of heavy mitochondria in cold-acclimated rat adipocytes as opposed to controls (unpublished results). This has been reported also by Lusena and Decopas [43].

Our data show that cold acclimation induces alterations of the lipid composition at the adipocyte membrane level in the rat.

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